Virology 394 (2009) 19–27

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

An evaluation of transmission routes for low pathogenicity avian influenza virus among chickens sold in live bird markets

Karen S. Yee [a], Tim E. Carpenter [a], Thomas B. Farver [b], Carol J. Cardona [b,c,*]

[a] Center for Animal Disease Modeling and Surveillance (CADMS), Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA, USA
[b] Department of Population Health and Reproduction, University of California Davis, CA, USA
[c] Veterinary Medicine Extension, University of California Davis, CA, USA

ARTICLE INFO

Article history:
Received 3 February 2009
Returned to author for revision
16 March 2009
Accepted 7 August 2009
Available online 10 September 2009

Keywords:
Low pathogenicity avian influenza
Live bird market
H6N2
Virus transmission
Poultry
Poultry diseases
Indirect contact

ABSTRACT

Many theories about the modes of avian influenza virus (AIV) transmission have been proposed, but few have been quantified, and none within a flock or live bird market (LBM) setting where birds are often kept in stacked cages. We describe a novel experimental design and the results collected for the purpose of estimating transmission rates specific to the potential modes of AIV transmission within an LBM. Chickens of the strains and ages found in California LBMs were inoculated with low pathogenicity AIV H6N2. Aerosol exposure was found to be the most important route of transmission for this H6N2 AIV. The handling of infectious chickens resulted in the transmission of H6N2 AIV, though the virus was not detectible by rRT-PCR. Chickens with fecal exposure to infected birds (median = 8.0 DPI) had detectable virus earlier than in those with aerosol exposure only (median = 10.0 DPI). Changes in the hemagglutinin sequence were not found to be associated with oropharyngeal or cloacal shedding in this study.

Introduction

Avian influenza viruses (AIVs) are enveloped, single-stranded, negative-sense RNA viruses of the *Orthomyxoviridae* that can cause disease in humans, poultry, and wild birds (Cardona et al., 2009; Swayne and Halvorson, 2003). Despite the involvement of live bird markets (LBMs) in many AIV epidemics worldwide (Senne et al., 1992; Swayne and Halvorson, 2003; Webster, 2004) little is known about how AIV spreads within an LBM. A better understanding of AIV transmission and spread is necessary to plan efficient and successful control and eradication programs. In previous studies of direct and indirect contact AIV transmission, (Alexander et al., 1978; Perez et al., 2003; Samadieh and Bankowski, 1971) either insufficient numbers of birds were used, or study designs prevented accurate and precise estimations of transmission rates.

Though AIV transmission has been demonstrated by direct contact in other studies (Alexander et al., 1986; Lu and Castro, 2004; van der Goot et al., 2003), previous studies have not attempted to measure transmission rates by indirect routes in environments such as LBMs, where birds are usually kept in stacked cages and most AIV transmission will be via indirect contact, such as aerosol and fecal exposures. The roles of aerosol and fecal exposures have been examined in other studies (Alexander et al., 1978; Perez et al., 2003), but indirect exposures in large populations and their combined effect on AIV transmission have not been examined. Another mode of indirect exposure for birds in LBMs is via contaminated fomites, like the gloves, aprons, and rubber boots worn by LBM employees while handling birds. Tiwari et al. (2006) demonstrated that AIV virus can remain viable on non-porous materials after contamination. In that study, materials such as latex gloves, gumboots, cotton fiber, feathers and plastics were contaminated with a 6.3×10^6 tissue culture infections dose $(TCID)_{50}$/ml of AIV H13N7, and approximately 10^2–10^3 infectious virus particles were recovered 0–72 h post-contamination. However; an experiment to estimate the transmission of AIV surviving on these materials to chickens was not conducted.

This study was designed to collect the data needed to quantify the transmission rate of AIV among birds in an LBM, which are commonly kept in cages and do not have homogeneous contacts. We used chickens of the types commonly sold in LBMs, with a novel study design mimicking the direct and indirect routes common in an LBM setting, to study the transmission of A/chicken/California/1772/02 (H6N2).

Results

No signs of disease were observed prior to necropsy in any birds in either trial. Splenomegaly ($n = 21$ and 20 chickens in trials 1 and 2, respectively) was the most common lesion observed in infected

* Corresponding author. Department of Population Health and Reproduction, Veterinary Medicine Extension, University of California Davis, One Shields Avenue, Rm. 1383 Surge III, CA 95616, USA. Fax: +1 530 752 7563.
E-mail address: cjcardona@ucdavis.edu (C.J. Cardona).

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.08.017

Table 1
Daily rRT-PCR and log 2 HI results for trials 1 and 2.

Trial 2—without trays between cages				rRT-PCR (DPI)															HI log2 (DPI)	
	Cage	Handling order	Contact type	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	0	14
Stack 1	A	1	Aerosol	–	–	–	–	–	–	–	–	r	–	r	r	r, c	r	–	–	0	6
			Aerosol	–	–	–	–	–	–	–	–	r	r	r	r	r	r	–	–	0	7
			Aerosol	–	–	–	–	–	–	–	–	–	r	r	r	r	–	c	–	0	0
			Aerosol	–	–	–	–	–	–	–	r	r	r	–	r, c	r, c	r	c	–	0	10
			Aerosol	–	–	–	–	–	–	–	–	r	r	r	r, c	r, c	r, c	c	–	0	8
	Z	4	Inoculated	–	–	r	r	–	r	r	–	–	–	–	–	–	–	–	–	0	11
			Inoculated	–	–	–	r	r	r	r	–	–	–	–	–	–	–	–	–	0	10
			Inoculated	–	–	–	r	r	r	r	–	–	–	–	–	–	–	–	–	0	10
			Inoculated	–	–	–	r	r	r	r	–	–	–	–	–	–	–	–	–	0	10
			Inoculated	–	–	–	r	r	r	r	r, c	c	–	–	r	–	c	–	–	0	11
	B	8	Aerosol, fecal	–	–	–	–	-	r	r	r	r	r	r, c	r	–	-	–	–	0	13
			Aerosol, fecal	–	–	–	–	r	r	r, c	r, c	–	–	–	–	–	–	–	–	0	11
			Aerosol, fecal	–	–	–	–	–	r	r	r	r	c	–	–	–	–	–	–	0	12
			Aerosol, fecal	–	–	–	–	–	–	–	r	r	r	r	–	c	–	–	–	0	12
			Aerosol, fecal	–	–	–	–	–	r	r	r	r, c	r	r, c	–	–	–	–	–	0	12
	C	3	Aerosol, fecal	–	–	–	–	–	–	–	–	r	r	–	r, c	r, c	c	–	–	0	7
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	–	–	r, c	–	c	–	0	5
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	–	r	–	–	–	–	0	9
			Aerosol, fecal	–	–	–	–	–	–	–	–	r	r	–	r, c	-	r	–	–	0	10
			Aerosol, fecal	–	–	–	–	–	–	–	r	r	r	r	r	–	–	–	–	0	11
Stack 2	D	5	Aerosol	–	–	–	–	–	–	–	–	–	–	r	r	r	r	r	-	0	0
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	r	r	–	–	0	0
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	r	r	r	–	–	0	0
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	r	r	–	–	0	0
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	9
	E	2	Aerosol, fecal	–	–	–	–	–	–	–	–	–	c	r, c	r, c	r, c	r	–	–	0	10
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	r	r	r, c	r	r	–	–	0	8
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	–	r	r, c	r, c	–	–	0	0
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	–	r	r	r	c	–	0	0
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	-	r	r	r	r	–	0	0
	F	7	Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	r	-	r	r	r, c	–	0	0
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	r	-	r, c	r, c	r, c	–	0	0
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	r	-	r	r	r	–	0	0
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	-	-	r, c	r, c	r, c	c	0	6
			Aerosol, fecal	–	–	–	–	–	–	–	–	–	–	–	–	r	r	–	–	0	7
Room 2	G	6	Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	9
			Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0
			Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	8
			Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0
			Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	3

Trial 2—with trays between cages				rRT-PCR (DPI)															HI log2 (DPI)	
	Cage	Handling order	Contact type	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1	14
Stack 1	A	1	Aerosol	–	–	–	–	–	–	–	r	r	r	r	r	–	–	–	c	0	10
			Aerosol	–	–	–	–	–	–	–			r	r	r	–	–	–	–	0	11
			Aerosol	–	–	†														0	-
			Aerosol	–	–	–	–	–	–	r	–	r, c	r ,c	r, c	c	–	–	c	c	0	11
			Aerosol	–	–	–	–	–	–		r	†								0	-
	Z	4	Inoculated	–	–	–	–	–	–	r	–	–	–	r	–	–	–	–	–	0	11
			Inoculated	–	r	r	r	r	r	–	–	–	–	-	–	–	r	–	–	0	10
			Inoculated	–	–	r	r, c	–	c	r, c	-	c	c	c	–	–	–	–	c	0	12
			Inoculated	–	r	r	r	r	r	–	–	–	–	–	–	–	–	–	–	0	11
			Inoculated	–	r	r	r	-	r	–	–	–	–	–	–	–	–	–	–	0	11
	B	8	Aerosol	–	–	–	–	–	–	–	–	r	r	r	–	–	r	–	–	0	12
			Aerosol	–	–	–	–	–	–	–	–	–	c	–	–	–	r	r	r	0	7
			Aerosol	–	–	–	–	–	–	–	–	r	c	r, c	r	–	–	–	–	0	10
			Aerosol	–	–	–	–	–	–	–	–	–	–	r	r	r	r, c	r	–	0	7
			Aerosol	–	–	–	–	–	–	–	–	–	r	r	r	–	r	c	r	0	11
	C	3	Aerosol	–	–	–	–	–	–	–	–	–	–	r, c	r	–	–	–	–	0	6
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	r	0	7
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	r	r	0	8
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	r	0	10
			Aerosol	–	–	–	–	–	–	–	–	r	r	r, c	r, c	–	–	c	c	0	11
Stack 2	D	5	Aerosol	–	–	–	–	–	–	–	–	–	r	r	r	–	r, c	r, c	r, c	0	9
			Aerosol	–	–	–	–	–	–	–	–	r	r	r	–	–	c	c	c	0	10
			Aerosol	–	–	–	–	–	–	–	–	r	r	–	r	–	r	–	–	0	10
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	r	–	r	–	r	0	10
			Aerosol	–	–	–	–	–	–	–	–	–	–	r	r	–	r	–	–	0	0

Table 1 *(continued)*

Trial 2—without trays between cages				rRT-PCR (DPI)															HI log2 (DPI)	
	Cage	Handling order	Contact type	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	0	14
	E	2	Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	r	0	9
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	r	–	r, c	0	10
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	r	0	10
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	r	r	0	10
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	c	r	0	10
	F	7	Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	r	–	r	0	7
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	c	–	r	–	r	0	8
			Aerosol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	r	r	0	10
			Aerosol	–	–	–	–	–	–	–	–	–	–	r	–	r	r	r, c	c	0	10
			Aerosol	–	–	–	–	–	–	–	–	–	–				r, c	c	c	0	9
Room 2	G	6	Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	10
			Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0
			Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	7
			Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	6
			Handling only	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	6

A positive rRT-PCR oropharyngeal sample is noted by an "r" and a positive rRT-PCR cloacal samples is noted by a "c." Birds that died before the end of the trial is noted by a "†".

chickens necropsied on day 16 for both trials. In trial 1, there were six chickens with yolk peritonitis, two with pale kidneys, four with regressing ova, and one with lung congestion. In trial 2, there were seven chickens with airsaculitis, two with hepatomegaly, two with lung congestion, and one with yolk peritonitis. There was no detected correlation between the appearance of lesions and virus shedding patterns, although the types of lesions were similar to those observed in commercial egg-laying chickens infected with H6N2 AIV in California (Kinde et al., 2003). During trial 2, a chicken located in cage A died on day 3 and another on day 9. There were no significant lesions observed in the chicken that died on day 3. Pale kidneys and splenomegaly were observed in the chicken that died on day 9. No significant lesions were observed in 15 and nine chickens in trials 1 and 2, respectively.

Table 1 shows the daily number of AIV-infected chickens detected by rRT-PCR and their HI responses prior to inoculation and 14 days post-inoculation (DPI) in both trials. Neither AIV nor AIV antibodies were detected prior to inoculation of the index birds. In trial 1 (no trays between cages), AIV was first detected in the inoculated birds (cage Z) 2 DPI, and 4 DPI in chickens in cage B. In trial 2 (trays between cages), AIV was first detected in the inoculated birds (cage Z) 1 DPI, and 6 DPI in chickens in cage A. AIV was detected in the inoculated birds for 4–8 and 5–9 days for trials 1 and 2, respectively. In exposed chickens located in cages A-E, AIV was detected for 3–8 days in trials 1 and 2. The daily median and range of virus particle numbers detected are shown in Fig. 2. In trial 1, 10^2–10^3 virus particles were detected from at least one bird in every cage in room 1. An estimated $10^{3.6}$ virus particles were the most detected in any bird in trial 1. In trial 2, 10^6 virus particles were detected in a bird in cage C once and $10^{5.5}$ virus particles was detected in an inoculated bird once. In the remaining five cages in room 1, 10^2–10^3 virus particles were detected from at least one bird in every cage.

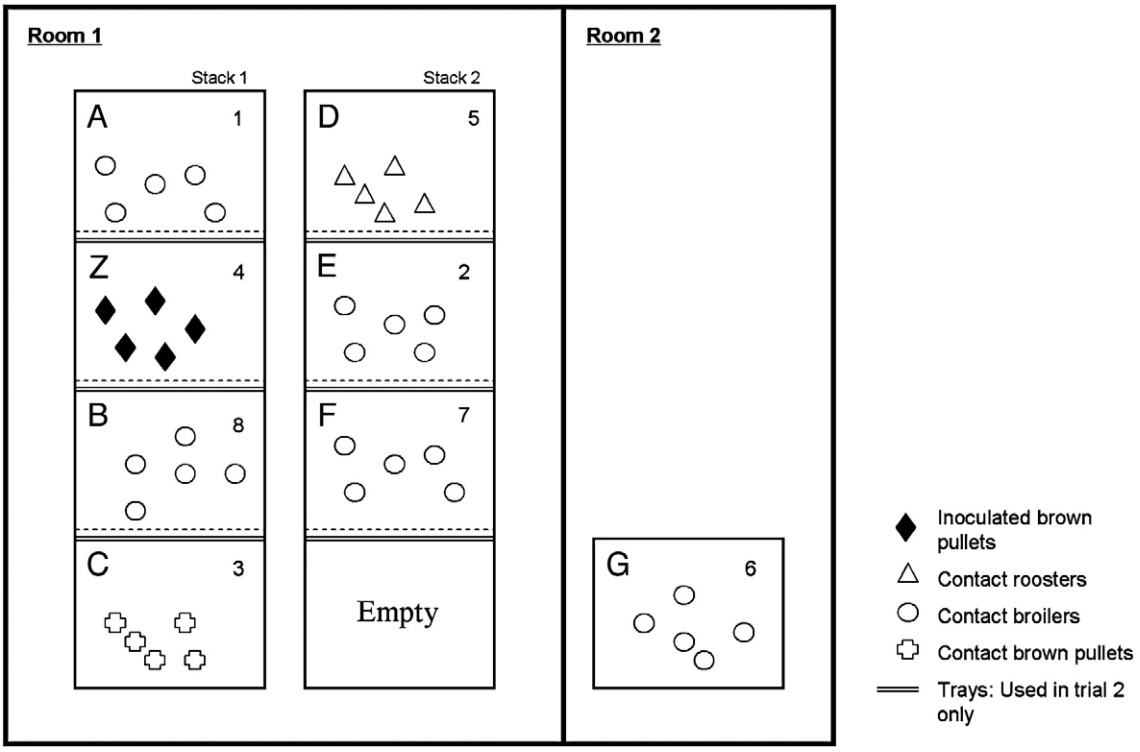

Fig. 1. Experimental design for transmission of LPAIV H6N2 between chickens in stacked cages labeled A–G. The handling order is numbered 1–8.

Table 2
Median time to detection and multiple comparisons results for all cages in trials 1 and 2.

Cage	Number of chickens/cage	Median time to detection (days)	Range (days)	Mean rank
Trial 1: no trays between cages				
A	5	8.0	7–9	$10.50^{\alpha\beta}$
B	5	5.0	4–7	3.20^{α}
C	5	8.0	7–12	$13.00^{\alpha\beta}$
D	5	11.0	10–12	24.90^{β}
E	5	11.0	9–11	19.80^{β}
F	5	10.0	10–12	22.10^{β}
G	5	N/A	–	–
Trial 2: trays between cages				
A	4*	7.0	7–9	4.88^{α}
B	5	9.0	8–10	9.70^{α}
C	5	14.0	8–15	$19.90^{\alpha\beta}$
D	5	9.0	6–11	$10.00^{\alpha\beta}$
E	5	14.0	13–14	24.40^{β}
F	5	13.0	11–14	$19.10^{\alpha\beta}$
G	5	N/A	–	–

Mean ranks without a superscript in common are significantly different with a 5% level of significance over all comparisons using the nonparametric multiple comparison procedure. *Cage A, one bird died on day 2 of the experiment, another chicken died on day 9, after AIV detection by RT-PCR.

The median first detection by rRT-PCR was 9 and 10 DPI for birds in trials 1 (without trays) and 2 (with trays between the cages), respectively. In both trials, AIV was detected in 29 of the non-inoculated birds housed in room 1; however, this occurred 3 days earlier in trial 1 than in trial 2. The Peto-Gehan Wilcoxon test comparing the time to detection in trial 1 and trial 2 was non-significant ($p = 0.071$).

All sequences were compared to A/turkey/Massachusetts/3740/1965. There was no differences in the amino acid sequences of A/chicken/CA/1255/2002 (H6N2) and eight samples from this study (five from trial 1 and 2 from trial 2). The HA cleavage site amino acid sequence in all the sample sequences and A/chicken/CA/1255/2002 (H6N2) was PQIATR↓G. This cleavage site was PQIETR↓G for A/chicken/California/431/2000 (H6N2). Amino acids at position 222 have been experimentally shown to play a role in sialic acid cell receptor recognition for H6 viruses (Gambaryan et al., 2008). All HA amino acid sequences from samples, A/chicken/California/431/2000 and A/chicken/CA/1255/2002 have Val222. There were three differences in the nucleotide sequences from the experimental samples that translated into amino acid changes at positions 60, 73 and 206. Most samples had Cys60 except for two samples from birds housed in stack one and one sample from a bird housed in stack 2 in trial 1 had Gly60. All sequenced samples from trial 1 and three samples from trial 2 had Cys73. Four samples from trial 2 (three in stack 1 and one in stack 2) had Ser73. One sample in trial 2 had Val206 and all other samples had Ala206. There were no statistical difference between the number of oropharyngeal and cloacal samples that had Gly60 or Cys60 ($p = 0.494$).

Comparison of AIV detection by rRT-PCR among the cages-trial 1

To evaluate the impact of fecal exposure on transmission to the birds in lower cages, trays were not placed between the cages in this trial. In this trial, the first AIV transmission was detected in cage B, located directly beneath cage Z containing the inoculated birds (Fig. 1). Time to detection was significantly different ($p = 0.001$) by cage (Table 2). Pairwise comparisons of time to AIV detection in each cage showed that detection of AIV in cage B (median = 5.0 DPI) was significantly earlier than in cages located in the adjacent stack (cage D median = 11.0 DPI; cage E median = 11.0 DPI; and cage F median = 10.0 DPI). Median time to detection for cage B was earlier than, but not significantly different from, cages located within the same stack (cages A and C median = 8.0 DPI). No AIV was detected by rRT-PCR in birds located in the room 2 (cage G), which were handled after the index case and other subsequently infected birds, although antibodies were detected in two chickens in cage G at 14 DPI.

Comparison of AIV detection by rRT-PCR among the cages-trial 2

Trays were placed below the cages in trial 2 to evaluate the effect of eliminating fecal exposure to birds located below other cages.

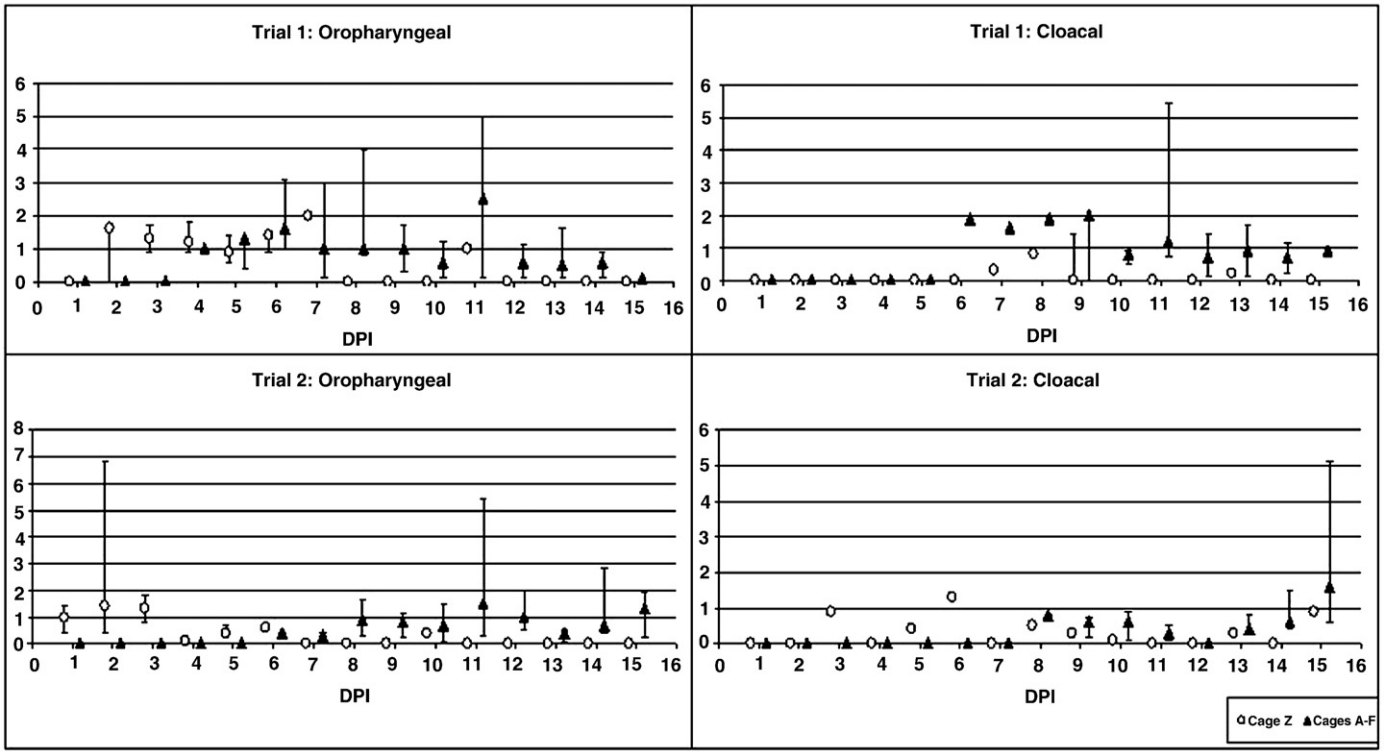

Fig. 2. Median and range of estimated virus particles ($\log_{10}$) detected for all rRT-PCR samples.

Median time to detection for the chickens grouped by cages differed significantly ($p = 0.002$, $df = 5$). The first detection of AIV by rRT-PCR was in a chicken housed in the top cage of the second stack (cage D). Pairwise comparisons of time to AIV detection for each cage showed that detection of AIV in cage A, located directly above the inoculated birds (median = 7.0 DPI), was significantly earlier than that for birds in cage E (median = 14.0 DPI), located directly across from the inoculated birds. No other comparisons were statistically significantly different. No AIV was detected by rRT-PCR in birds located in room 2 (cage G), although antibodies were detected in four chickens in cage G on 14 DPI.

AIV transmission in room 1

Detection of AIV was significantly earlier in chickens housed in the same stack as inoculated birds (stack 1) than in birds housed in the adjacent stack (stack 2) in both trials ($p < 0.001$ and $p = 0.031$ for trials 1 and 2, respectively) (Fig. 3). The time to detection by rRT-PCR for stack 1 in trial 1 was significantly earlier than AIV detection in stack 1 during trial 2 ($p = 0.017$). A statistical comparison of time to detection in stack 2 in trials 1 and 2 was performed in two steps as the rRT-PCR detection curves intersect at day 11, and the intersection of the detection curves violates the test assumptions that the differences in these curves are proportional. Time to detection in stack 2 for days 1–10 was not significantly different between trials 1 and 2 ($p = 0.154$). Time to detection in stack 2 for days 11–15 was significantly earlier for trial 1 than trial 2 ($p = 0.037$).

Fecal exposure

There was a non-significant difference ($p = 0.068$) in time to detection by rRT-PCR between the two trials using the Wilcoxon rank-sum test. In trial 1 time to detection was compared for cages that had 0, 1, and 2 cages physically above them with birds potentially shedding AIV (Table 3). Cages A and D did not have fecal exposure. Cage E was considered as having no fecal exposure since AIV was not

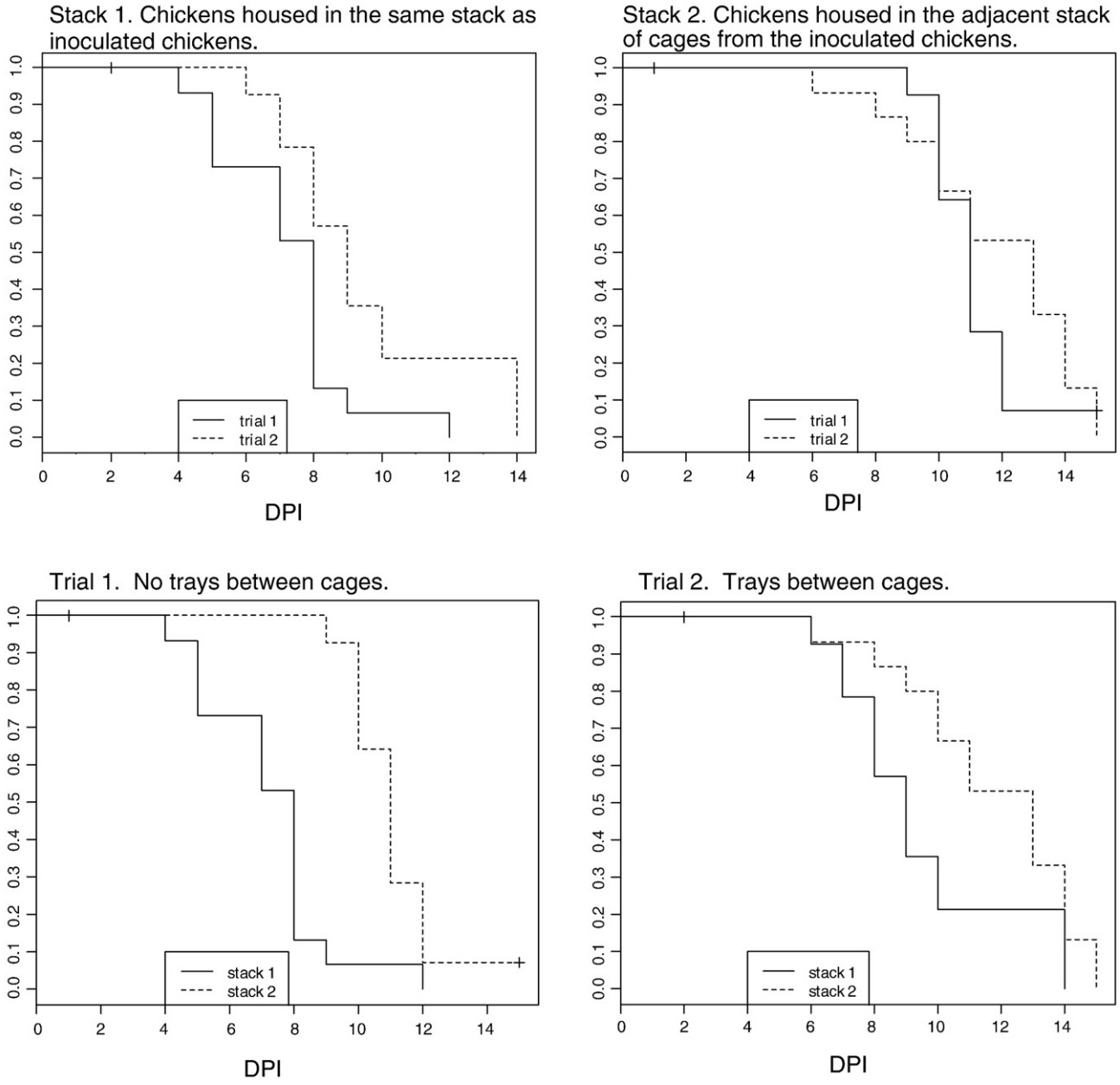

Fig. 3. Daily proportion of chickens in room 1 with no detectable AIV infection by RT-PCR.

Table 3
Median time to AIV detection and Kruskal–Wallis mean rank scores for groups based on their fecal exposure.

Fecal exposure level	Number of susceptible chickens	Median time to detection (DPI)	Range (DPI)	Mean rank
Trial 1: no trays between cages				
None				
(A, D, E)	15	10.0	7–16	18.23
One cage above				
(B, F)	10	8.5	5–12	12.65
Two cages above				
(C)	5	8.0	7–12	13.00
Trial 2: trays between cages				
A, D, E	14	10.0	6–16	13.69
B, F	10	10.0	8–14	14.40
C	5	14.0	8–15	19.90

Trial 1, $p=0.228$, $df=2$. Trial 2, $p=0.355$, $df=2$.

detected in chickens in the cage above it (cage D) prior to the chickens in cage E. Cages B and F each had one cage with AIV-positive chickens above them prior to shedding virus (Fig. 1). There were two cages above cage C in which AIV was detected prior to chickens in cage C. Median time to AIV detection in cages with no fecal exposure (cages A, D and E) were not significantly different ($p=0.228$) than time to AIV detection in cages that had fecal exposure (cages B, C and F). Comparing the same groups (cages A, D, E vs. cages B, C, and F) in trial 2, there was also no significant difference ($p=0.355$) in time to detection between the two groups of cages.

Expected and observed patterns of transmission by handling

There was no detectable pattern of AIV transmission by handling infected chickens prior to susceptible chickens in either trial in room 2. When analyzing median times to detection grouped by cage in room 1, the mean ranking scores for the cages did not follow the order of handling. Additionally, chickens handled before infected/inoculated chickens did not have higher mean ranks than those handled after infectious/inoculated chickens. In trial 1, where trays were not present between the cages, those in the same stack as inoculated birds (cages A, B, and C) had lower median times to detection than the other stack of cages. In trial 2, where trays were placed between the cages, those cages that were closest to inoculated birds (cages A, B and D) had lower median times to detection than the other cages in the same room. No AIV was detected by rRT-PCR in chickens placed in room 2 (cage G), which were handled after inoculated and subsequently infected birds; however, antibodies were detected in two and four birds in trials 1 and 2, respectively, in cage G at 14 DPI, indicating there was some transmission of AIV between birds by handling alone.

Discussion

Our results illustrate differences in AIV transmission by different exposure routes of H6N2 AIV, which caused outbreaks in Southern California poultry in 2000–2005 (Kinde et al., 2003; Shivaprasad et al., 2004; Woolcock, Suarez, and Kuney, 2003). Since the characteristics of different AIV subtypes and genotypes are so diverse, repetition of this experiment could yield different results. However, our results indicate that aerosol exposure is an important mode of AIV transmission among chickens in LBM settings. In both trials, cages in closest proximity to the inoculated chickens, or housed within the same stack of cages as the inoculated chickens, had the lowest median detection time. The direction of the air exchange rates direction did not appear to have an effect on AIV transmission in this experiment. In a separate transmission experiment conducted using the same virus (Yee, Cardona, and Carpenter, 2009), the air flow was in the opposite direction to that in this experiment and there were no detectable differences in AIV transmission between the two studies.

Among the rRT-PCR-positive samples selected for virus isolation and HA gene sequencing, there were no significant genetic or amino acid sequence changes associated with receptor binding. There are two amino acids that differ between trials 1 and 2 at positions 60 and 73. These amino acid positions are not known to be involved in cell receptor recognition and the significance of these differences are not known. Four of the seven samples selected for full-length HA sequencing had Ser73 that was not found in any sample sequences from trial 1, nor Ser73 found in samples collected from chickens infected through direct contact in another transmission study with the same virus (Yee, Cardona, and Carpenter, 2009). This difference in the sampled viruses between trials 1 and 2 may be related to differences in transmission characteristics between the two trials.

In a study conducted by Gambaryan et al. (2008), Ala222 in H6 viruses was demonstrated to have a major role in binding to duck, gull, and chicken sialic acid receptors. The virus used in this study, A/chicken/California/1772/02(H6N2), and the viruses used in the amino acid alignment, A/chicken/California/431/2000(H6N2) and A/chicken/CA/1255/2002(H6N2), have Val222. Although consistently found, this change to a similar (non-bulky) amino acid, may have limited impact on function (Gambaryan et al., 2008) and because it is consistently found in the inoculating virus, viruses of the same lineage and in viruses isolated in both trials, there are no indications that this amino acid change was selected in this trial. The amino acids in cleavage sites for the virus used in this experiment and A/chicken/CA/1255/2002(H6N2) also differs from contemporary Asian-origin strains of H6N2 and A/chicken/California/431/2000(H6N2) substituting alanine for glutamic acid (Chin et al., 2002). A single change in the amino acid composition of the cleavage site appeared consistently in the viruses isolated from birds to which there was likely fecal transmission suggesting it may have been selected by the route of transmission, although this could not be established with the design of the current study. Further studies to experimentally examine the role of this change in viral transmission, tropism and shedding patterns are planned.

Previous studies on the transmission of AIV in turkeys did not result in detectable transmission between turkeys in different cages (Samadieh and Bankowski, 1971). Based on results of that study and others (Perez et al., 2003; Samadieh and Bankowski, 1971; van der Goot et al., 2003), we did not expect aerosol exposure to be the major vehicle of AIV transmission among chickens in this experiment. Some experimental replications by van der Goot et al. using LPAIV, A/Chicken/Pennsylvania/21525/83 (H5N2) did not result in transmission to chickens in direct contact with inoculated birds (van der Goot et al., 2003). Results of previous AIV indirect contact transmission studies between Japanese quail and chickens have been mixed. Perez et al., in their studies of Asian H9N2 AIVs, observed transmission of A/Quail/Hong Kong/A28945/88 (H9N2) to susceptible quail and chickens as a result of fecal but not aerosol exposure (Perez et al., 2003). It is possible that transmission of the AIV subtypes studied by Perez would have been observed if the experiment were extended beyond 7 days post-inoculation, as we observed AIV transmission within 6–14 DPI in birds with aerosol exposure only.

The effect of fecal transmission was difficult to assess because the differences in time to detection between the two trials ($p=0.071$) and the comparison of trials 1 and 2 for the chickens housed in room 1 ($p=0.068$) were marginally non-significant. However, daily AIV prevalence was always higher in trial 1 than in trial 2, suggesting that fecal exposure did result in transmission to susceptible birds, or it resulted in the exposure of birds to more virus, which may extend their periods of shedding and the amount of virus shed (C. J. Cardona, unpublished data). Additionally, the initial transmission beyond cage Z (inoculated birds) in trial 1 (no trays) implied that fecal exposure played an important role since it occurred in a chicken located

directly below the index cases (cage B), whereas in trial 2 (trays placed between the cages), AIV spread was first detected in a cage located above the inoculated birds (cage A). Based on our results comparing the Kaplan–Meier survival curves of AIV detection by rRT-PCR in room 1, it appears that placing trays between cages, thus preventing fecal exposure, delayed transmission between cages within the same stack. AIV detection in chickens housed in the stack of cages adjacent to the inoculated birds (stack 2) was very different in trial 1 than in trial 2. While there were no significant differences in AIV detection for the first 10 days in stack 2 in either trial ($p=0.154$), detection of AIV 10 DPI was significantly earlier in trial 1 than in trial 2 and may explain the marginally non-significant results between the trials.

The purpose of the handling assessment was to determine the role of fomites in the spread of AIV among chickens in an LBM. This study suggests that fomites commonly used in LBMs may play a role in transmission of AIV, which may go undetected by active surveillance efforts for some period of time using rRT-PCR only. We observed evidence of mechanical transfer of H6N2 AIV by handling infectious birds prior to handling susceptible birds housed in another room within the 16-day time period. There was no difference in detection time by rRT-PCR of chickens following the handling pattern, nor was there a difference in detection time in birds within the room that were handled prior to the inoculated/infectious birds and birds handled after the inoculated/infectious birds. Since infection was only detected by antibody response in room 2, it was not possible to determine more closely when AIV infection occurred in these chickens.

In the Tiwari et al. (2006) experiment, 10^2–10^3 virus particles were found on contaminated non-porous materials. Previous experiments using a different genotype of H6N2 AIV have shown that fewer than 10^6 EID_{50}/ml will not consistently cause an infection as detected by rRT-PCR in intranasally inoculated chickens (C.J. Cardona, unpublished data). These previous experiments support our results in which AIV was not detected in the chickens housed in room 2 by rRT-PCR, but infection was detected by antibody response. It is possible that there was an insufficient dose of virus on the gloves, apron, and boots to transmit and produce a fulminant infection resulting in shedding that could be detected by rRT-PCR. These results are useful since active surveillance in LBMs are conducted using rRT-PCR on oropharyngeal and cloacal swab samples (Yee et al., 2008). It is unknown if these birds could play a part in spreading AIV to other susceptible birds, and increasing the frequency of cleaning these fomites may prevent or reduce AIV transmission through this route.

While the placement of trays below the cages did not prevent the spread of AIV between birds in room 1, using trays may nonetheless have a significant effect on reducing viral spread within LBMs. Chickens usually spend from 1 to 7 days in an LBM in Southern California, while shipments of new birds to a market arrive 2–7 times per week (Yee et al., 2008). Therefore, if there is an infection in the market, the number of initial susceptible and infectious birds present is not fixed as it was in our experimental study.

Data and findings from this analysis can be used in a mathematical model to quantify the probability and rate of LPAIV transmission. Parameters from this model can then be used in simulation models to determine the spread and possible persistence of H6N2 AIV in LBM settings. These simulation models can account for the physical layout of an LBM, exposure routes, and the constant cycling of additions (by supplier deliveries) and removal (by custom slaughter sales) of birds, which are all variables that can affect disease transmission. A simulation model can also evaluate the effectiveness of control and prevention strategies, such as active surveillance, by estimating the frequency and number of swabs necessary to detect infection in a given period of time post-exposure, or the frequency of "day of rest" cleaning and depopulation activities that are conducted in LBMs in Hong Kong and Southern California (Kung et al., 2003; Yee et al., 2008).

Materials and methods

Virus

The virus used to inoculate birds in this study, A/chicken/California/1772/02, H6N2 (hereafter referred to as H6N2 AIV), was isolated from commercial egg laying chickens in Southern California in 2002. A closely related strain, A/Quail/California/KSY1031/2005, H6N2, was isolated from an outbreak in Southern California LBM in 2005 (K.S. Yee, unpublished data).

Animals

Birds were acquired through an LBM poultry supplier in California. The supplier participates in active and passive surveillance for AIV as a part of the California Custom Slaughter LPAIV Control Program (Yee et al., 2008). There were no AIV infections detected in any of the supplier's flocks immediately prior to or during the experimental period (C.J. Cardona, unpublished data.). Each of the chicken strains used in this experiment are of the types and strains sold in California LBMs (Yee et al., 2008) and were market age at the time of placement. Brown pullets ($n=10$) and roosters ($n=5$) were 8–24 weeks of age and broiler hens ($n=25$ in each trial) were 6–8 weeks of age.

Experimental procedures

The equipment used and feeding, watering, and lighting schedules were based on the most common practices in Southern California LBMs (Yee et al., 2008). Collectively, thirty susceptible chickens were placed five to a cage (cages A through F) in the same room (room 1), together with five inoculated chickens in another cage (cage Z) for a total of seven cages. Individual cages were arranged as two stacks of four cages, with stacks approximately six inches apart. One cage located on the bottom right was left empty. Broiler hens were placed in cages A, B, E, and F, brown pullets were placed in cage C, and roosters were placed in cage D (Fig. 1). Each cage measured 36"×18"×18", and chickens in one cage did not have direct beak-to-beak contact with birds placed in other cages. Additionally, five broiler hens were placed in one cage (cage G) isolated in another room (room 2) to determine the role of fomites alone in transmission.

The first trial was conducted without trays between stacked cages. After a complete cleaning and disinfection of the room, cages, and fomites, a second trial was conducted with trays inserted between the stacked cages. At the beginning of each trial, the five brown pullets in cage Z, located second from the top in the stack of four cages, were removed from their cage and inoculated intranasally with 10^7 EID_{50} of H6N2 AIV on day 1 in room 1 (shown in Fig. 1). The inoculated birds were immediately placed back into cage Z. Each bird in both trials was observed daily for clinical signs of disease. Necropsy was performed on the day of death if birds died before the end of the experiment, and on day 16 on all remaining birds.

During the trials, handlers wore a hycar rubber apron, rubber gloves, and rubber boots, which were stored in room 1 where the birds in the stacked cages were kept. This was designed to simulate handling conditions that occur in southern California LBMs, and the protective clothing were intended to serve as fomites for transferring AIV between the cages, rooms, and individual birds. Oropharyngeal and cloacal swab samples were collected daily from all birds for 16 days. Blood was collected from each bird at days 0 and 14. The order of sample collection by cage number was: A, E, C, Z, D, G, F, and B (shown in Fig. 1). Thus, three cages of chickens (cages A, E, and C) were handled prior to handling the inoculated chickens (cage Z). Samples were collected from each caged group using an order that could be distinguished from the expected patterns of spread between cages via fecal and aerosol routes of transmission.

There were three patterns of AIV detection expected if handling the birds effectively spread the virus. One expected pattern was that AIV would be detected in birds that were handled after the inoculated birds, but before the birds were handled prior to handling the inoculated birds (i.e., median time to detection for cages A, E, and C would be greater than cages D, G, F and B). The second expected pattern was that AIV detection would follow the handling order, so that cage D would have the lowest median time to detection, followed by cages G, F, and B. The third expected pattern was that the birds located in room 2 would become infected, since they were handled after the inoculated birds were handled.

In room 1, birds placed at the top of the stacks, and those in trial 2 (in which trays were placed between the cages) were exposed via aerosol exposure, but had no fecal exposure. Birds placed in room 2 were isolated from those in room 1 and had handler contact (a technician wearing the same apron, gloves, and boots) but no fecal or aerosol exposure. During both trials, technicians were unaware of the identity and location of the index cases.

RT-PCR and sequence analyses

All samples were tested for virus with real-time (r) RT-PCR following the methods published by Spackman et al. (2003). The same rRT-PCR test was run on known dilutions of the challenge virus to establish a standard curve that could be used to quantitate the number of virus particles in positive samples.

Virus isolation and HA sequence analyses

Fifteen rRT-PCR-positive samples were selected for full-length sequencing of the hemagglutinin (HA) gene. Eight rRT-PCR-positive samples from trial 1 and seven samples from trial 2 were selected for virus isolation. At least one sample from at least one chicken in each cage was selected to determine genotypic associated with adaptation, and the site of virus shedding (oropharyngeal and/or cloacal). Virus was grown and detected following standard methods (Swayne et al., 1998). Nine to 11 days old SPF chicken embryos (Charles River Laboratories, North Franklin CT) were inoculated with 150 µl of virus sample and incubated at approximately 37 °C for 72 h. The allantoic fluid was harvested after the embryos were chilled at 4 °C overnight and tested for hemagglutinating activity using 0.5% chicken red blood cells.

Viral RNA was extracted from the allantoic fluid harvested from infected embryonating chicken eggs using the Qiagen Viral RNA mini extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A two step RT-PCR was used to amplify the HA gene. RNA was transcribed using 0.5 µl (1 µg/µl) of Uni12 primer 5′-AGCAAAGCAGG-3′, 7 µl of viral RNA and incubating at 70 °C for 5 min and chilled to 4 °C. The reverse transcription reaction was amplified at 25 °C for 10 min and followed by extension at 42 °C for 1.5 h by MLV polymerase (Invitrogen, Carlsbad, CA) with the addition of dNTPs and RNase inhibitor. The reaction was terminated at 75 °C for 10 min at the end of the cDNA preparation step.

The full-length individual influenza genes were amplified using TaKaRa Taq PCR system (TaKaRa, Japan) according to manufacturer's protocol. For full-length HA genes from the cDNA were amplified with the HA gene specific primer described previously in Hoffmann et al. (2001) with modifications. Two internal H6 primers H6-576F (5′-TAYTTCTGGGGKGTRCAYCATCCW-3′) and H6-1098R (5′-ATTTTCA-TGRTGRTARCCATACCA-3′) were used to sequence the middle section of the HA gene. The amplified samples and primers were submitted to an outside facility for sequencing (Davis Sequencing, Davis, CA). The gene segments were assembled and consensus sequences were generated using VectorNTI ContigExpress in Invitrogen VectorNTI Advance 10 (Carlsbad, CA).

Nucleotide and amino acid sequences from all 15 viruses were aligned with the HA genes of A/chicken/California/431/2000(H6N2), A/chicken/CA/1255/2002 (H6N2), and A/turkey/Massachusetts/3740/1965(H6N2) in Geneious Pro (version 4.6.5, Biomatters Ltd, Auckland, New Zealand).

Serology

Hemagglutinin inhibition (HI) tests on blood samples were conducted using standard methods to confirm AIV-infection status prior to inoculation and at the end of the study period (World Organisation for Animal Health, 2005). Hemagglutination inhibition serum titers of 1:8 or greater were considered positive.

Statistical analysis

Descriptive statistics were performed on the day of first detection of AIV by rRT-PCR for birds in each cage. Times to detection for inoculated birds were not included in the analysis. The Wilcoxon rank-sum test was performed to compare median time to first detection by rRT-PCR between the two trials in SPSS (Release 10.0.0, September 1999). Kruskal–Wallis, a non-parametric analysis of variance procedure for ranked data, was used to test differences in time to detection by rRT-PCR of AIV between comparison groups (Kruskal and Wallis, Dec 1952). The mean rank of time to infection for each cage was compared to determine if there was a pattern of AIV spread among chickens, based on their proximity to inoculated birds or exposure type. Chickens were also grouped by their exposure to fecal material to assess differences in transmission times between birds with one, two, or no cage(s) of chickens shedding AIV above them. The non-parametric Bonferroni–Dunn procedure was used to identify significant ($\alpha < 0.05$ overall comparisons) group comparisons following each significant Kruskal–Wallis test (Daniel, 1990).

Life-tables and survival curves using the Kaplan–Meier method were created in R software (version 2.7.0) using the first day of detection by rRT-PCR for each bird to evaluate if the rate of transmission was significantly different given physical proximity to inoculated birds and fecal exposure. The statistical significance of time to AIV detection by rRT-PCR between birds in trials 1 and 2, as well as the differences in AIV detection between birds housed in each stack of cages in room 1, were evaluated using Peto-Gehan Wilcoxon of Kaplan–Meier survival curves (Harrington and Fleming, 1982).

Acknowledgments

We thank Drs. Jinling Li and Zeng-Qi Yang for their laboratory guidance and for performing the HI tests. We also thank Nicole L. Anchell, Nguyet Dao, Phuong Dao, and Sara Leisgang for providing technical and laboratory support. This work was supported by the Center for Animal Disease Modeling and Surveillance (CADMS) at the University of California Davis and funding was provided by the Avian Influenza Coordinated Agricultural Project, USDA/CSREES grant 2005-35605-15388, "The Prevention and Control of Avian Influenza in the United States."

References

Alexander, D.J., Allan, W.H., Parsons, D.G., Parsons, G., 1978. The pathogenicity of four avian influenza viruses for fowls, turkeys and ducks. Res. Vet. Sci. 24 (2), 242–247.
Alexander, D.J., Parsons, G., Manvell, R.J., 1986. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks, and quail. Avian. Pathol. 15, 647–666.
Cardona, C.J., Xing, Z., Sandrock, C.E., Davis, C.E., 2009. Avian influenza in birds and mammals. Comp. Immunol. Microbiol. Infect. Dis. 32 (4), 255–273.
Chin, P.S., Hoffmann, E., Webby, R., Webster, R.G., Guan, Y., Peiris, M., Shortridge, K.F., 2002. Molecular evolution of H6 influenza viruses from poultry in Southeastern China: prevalence of H6N1 influenza viruses possessing seven A/Hong Kong/156/97 (H5N1)-like genes in poultry. J. Virol. 76 (2), 507–516.

Daniel, W., 1990. Multiple comparisons. 2nd ed. In "Applied Nonparametric Statistics". PWS-KENT, Boston.

Gambaryan, A.S., Tuzikov, A.B., Pazynina, G.V., Desheva, J.A., Bovin, N.V., Matrosovich, M.N., Klimov, A.I., 2008. 6-sulfo sialyl Lewis X is the common receptor determinant recognized by H5, H6, H7 and H9 influenza viruses of terrestrial poultry. Virol. J. 5, 85.

Harrington, D.P., Fleming, T.R., 1982. A class of rank test procedures for censored survival data. Biometrika 69, 553–566.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146 (12), 2275–2289.

Kinde, H., Read, D.H., Daft, B.M., Hammarlund, M., Moore, J., Uzal, F., Mukai, J., Woolcock, P., 2003. The occurrence of avian influenza A subtype H6N2 in commercial layer flocks in Southern California (2000–02): clinicopathologic findings. Avian. Dis. 47 (3 Suppl), 1214–1218.

Kruskal, W., Wallis, W., Dec 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47 (260), 583–621.

Kung, N.Y., Guan, Y., Perkins, N.R., Bissett, L., Ellis, T., Sims, L., Morris, R.S., Shortridge, K.F., Peiris, J.S., 2003. The impact of a monthly rest day on avian influenza virus isolation rates in retail live poultry markets in Hong Kong. Avian. Dis. 47 (3 Suppl.), 1037–1041.

Lu, H., Castro, A.E., 2004. Evaluation of the infectivity, length of infection, and immune response of a low-pathogenicity H7N2 avian influenza virus in specific-pathogen-free chickens. Avian. Dis. 48 (2), 263–270.

Perez, D.R., Lim, W., Seiler, J.P., Yi, G., Peiris, M., Shortridge, K.F., Webster, R.G., 2003. Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. J. Virol. 77 (5), 3148–3156.

Samadieh, B., Bankowski, R.A., 1971. Transmissibility of avian influenza-A viruses. Am. J. Vet. Res. 32 (6), 939–945.

Senne, D., Pearson, J.E., Panigrahy, B., 1992. Proc. 3rd International Symposium on Avian Influenza. Madison, WI.

Shivaprasad, H., Charlton, B., Sommer, F., Cooper, G., Crespo, R., Woolcock, P., Castellan, D.M., 2004. Avian influenza H6N2 in California broiler chickens and turkeys-2002. "53rd Western Poultry Disease Conference".

Spackman, E., Senne, D.A., Bulaga, L.L., Myers, T.J., Perdue, M.L., Garber, L.P., Lohman, K., Daum, L.T., Suarez, D.L., 2003. Development of real-time RT-PCR for the detection of avian influenza virus. Avian. Dis. 47 (3 Suppl.), 1079–1082.

Swayne, D., Halvorson, D.A., 2003. Influenza, In: Saif, Y., Barnes, H., Glisson, J., Fadly, A., McDougald, L., Swayne, D. (Eds.), 11 ed. "Diseases of Poultry". Iowa State Press, Ames, IA, pp. 135–160.

Swayne, D., Senne, D., Beard, C., 1998. Avian influenza. In: Swayne, G.F., DE, Jackwood MW, Pearson, JE, Reed, WM, Reed, WM (Eds.), "Laboratory manual for the isolation and identification of avian pathogens". American Association of Avian Pathologists, Kennett Square (PA, pp. 150–155.

Tiwari, A., Patnayak, D.P., Chander, Y., Parsad, M., Goyal, S.M., 2006. Survival of two avian respiratory viruses on porous and nonporous surfaces. Avian. Dis. 50 (2), 284–287.

van der Goot, J.A., de Jong, M.C., Koch, G., Van Boven, M., 2003. Comparison of the transmission characteristics of low and high pathogenicity avian influenza A virus (H5N2). Epidemiol. Infect. 131 (2), 1003–1013.

Webster, R.G., 2004. Wet markets—a continuing source of severe acute respiratory syndrome and influenza? Lancet 363 (9404), 234–236.

Woolcock, P.R., Suarez, D.L., Kuney, D., 2003. Low-pathogenicity avian influenza virus (H6N2) in chickens in California, 2000–02. Avian. Dis. 47 (3 Suppl.), 872–881.

World Organisation for Animal Health, 2005. Chapter 2.7.12: Avian Influenza. In: OIE (Ed.), "Manual of Diagnostic Tests and Vaccines for Terrestial Animals". OIE.

Yee, K.S., Carpenter, T.E., Mize, S., Cardona, C.J., 2008. The live bird market system and low-pathogenic avian influenza prevention in Southern California. Avian. Dis. 52 (2), 348–352.

Yee, K.S., Cardona, C.J., Carpenter, T.E., 2009. Transmission of low-pathogenicity avian influenza virus of subtype H6N2 from chickens to Pekin ducks and Japanese quail (*Coturnix coturnix japonica*). Avian. Pathol. 38 (1), 59–64.